PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00, C07H 21/04, C12Q 1/68

A1

(11) International Publication Number: WO 98/13072

(43) International Publication Date: 2 April 1998 (02.04.98)

(21) International Application Number: PCT/US97/17320

(22) International Filing Date: 24 September 1997 (24.09.97)

(30) Priority Data:
60/026,958 24 September 1996 (24.09.96) US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: COMPOSITIONS FOR AND METHODS OF TREATING MULTIPLE DRUG RESISTANCE

(57) Abstract

Antisense oligonucleotides specific for the mdr1 gene are used to reverse multiple drug resistance in cancer cells *in vivo*. The oligonucleotides are administered as part of treatment regimen which includes anticancer chemotherapy.

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COMPOSITIONS FOR AND METHODS OF TREATING MULTIPLE DRUG RESISTANCE

Field of the Invention

The invention relates to antisense oligonucleotides, in particular to antisense oligonucleotides which prevent expression of the *mdr* gene, and to compositions for and methods of using such oligonucleotides to eliminate multiple drug resistance and render cells that are associated with disease conditions more susceptible to chemotherapeutic treatments.

Background of the Invention

The resistance of tumor cells to multiple chemotherapeutic agents, a phenomenon termed MDR, is a major obstacle to a successful cancer chemotherapy and has been closely associated with treatment failure. MDR is characterized by cross-resistance to a number of structurally and functionally unrelated drugs, due to overexpression of the *mdr1* gene product, P-glycoprotein.. Other mechanisms such as overexpression of glutathione S-transferase or mutation of topoisomerase II may also play a role in MDR. P-glycoprotein is a 170 KDA membrane glycoprotein that acts as an ATP-dependent efflux pump, increasing transport of various anticancer compounds out of cells and decreasing cellular accumulation of drugs and, thus, their efficacy. Anticancer drugs that are associated with P-170-mediated drug resistance include *Vinca* alkaloids (vinblastine and vincristine), anthracyclines (adriamycin), taxol, actinomycin D and mitomycin. Since there is a well established correlation between the expression of the *mdr1* gene and the activity of the P-170-mediated transport mechanism in human

tumors, compounds that can inhibit efflux by P-glycoprotein and enhance the accumulation of anticancer drugs might prove therapeutically useful. A variety of pharmacological agents, including verapamil, other calcium channel blockers, calmodulin inhibitors, cyclosporins, and steroid hormones, have been shown to interfere with P-glycoprotein function and to successfully reverse the MDR phenotype *in vitro*. However, the efficacy of these agents in animal studies and clinical trials has been disappointing due to dose-limiting toxicity and lack of specificity. Consequently, much effort is currently being directed toward developing compounds that inhibit P-170, reverse the MDR phenotype and sensitize tumor cells to conventional chemotherapeutic agents without undesired toxic effects.

Antisense oligodeoxynucleotides (ODNs) have attracted considerable interest as a tool to inhibit gene expression, raising the possibility that such compounds might be used to inhibit P-170 expression. Although antisense ODNs-mediated modulation of MDR in cultured cells has been described, to date no clear example of reversion of MDR by ODNs has been reported *in vivo*.

There remains a need for an effective *in vivo* treatment of individuals with cancer that is MDR. The remains a need for compositions and methods which can reverse MDR and render MDR cancer cells susceptible to drugs.

Summary of the Invention

The invention provides pharmaceutical compositions that comprise a pharmaceutically acceptable carrier and an antisense oligonucleotides specific for *mdr1* as hereinafter defined.

According to one embodiment, the oligonucleotide has a nucleotide sequence capable of forming a stable duplex with a portion of an mRNA transcript of the mdr1 gene.

The oligonucleotide is generally at least an 8-mer oligonucleotide, that is, the oligonucleotide is an oligomer containing at least 8 nucleotide residues, more preferably at least about 12 nucleotides. The preferred maximum size of the oligonucleotide is about 60 nucleotides, more preferably about 50 nucleotides. The oligomer is preferably an oligodeoxynucleotide. While oligonucleotides smaller than 12-mers may be utilized, they are statistically more likely to hybridize with non-targeted

sequences, and for this reason may be less specific. In addition, a single mismatch may destabilize the hybrid. While oligonucleotides larger than 40-mers may be utilized, uptake may become more difficult without specialized vehicles or oligonucleotide carriers. Most preferably, the oligonucleotide is a 15- to 40-mer oligodeoxynucleotide, more advantageously an 18- to 30-mer.

While in principle oligonucleotides having a sequence complementary to any region of the mdr1 mRNA find utility in the present invention, preferred are oligonucleotides capable of forming a stable duplex with a portion of the transcript lying within about 50 nucleotides (preferably within about 40 nucleotides) upstream (the 5' direction), or about 50 (preferably 40) nucleotides downstream (the 3' direction) from the translation initiation codon. Also preferred are oligonucleotides which are capable of forming a stable duplex with a portion of a mdr1 mRNA transcript including the translation initiation codon.

The invention is also a method for inhibiting expression of the mdr1 gene product P-glycoprotein (P-170) *in vivo*. Pharmaceutical compositions of the invention are administered to a patient and cells take up the antisense oligonucleotides. According to one preferred embodiment, the patient has cancer which has become drug resistant. The invention thus provides a method of treating multiple drug resistant malignant disease *in vivo* comprising administering to an individual of mdr1 antisense oligonucleotide sufficient to render the cancer cells susceptible to drug treatment, and administering an effective amount of a chemotherapeutic drug to eliminate cancer cells in the individual. In preferred embodiments, the malignant diseases treatable according to the invention is a blood disease such as leukemia.

Description of the Figures

Figure 1A and Figure 1B show the effect or *mdr1* [S]ODNs on HL-60/Vinc cell proliferation. The data in Figure 1A refers to cells were treated with sense (●) or antisense (■) *mdr1* [S]ODNs at a total dose of 200 μg/ml over 4 days (80 μg/ml at day 0 and 40 μg/ml from day 1 to day 3). Control cells (▲) were left untreated. Cell counts and viability were determined daily until the 8th day of culture. Representative of three different experiments with similar results. Fig. 1B shows data from cells were treated with scrambled (●) or antisense (■) *mdr1* [S]ODNs at a total dose of 360

μg/ml over 8 days (80 μg/ml at day 0 and 40 μg/ml from day 1 to day 7). Control cells (▲) were left untreated. Cell counts and viability were determined daily until the 8th day of culture. Representative of three different experiments with similar results. Both in (Fig.1A) and in (Fig. 1B) each value is an average ± standard error (S.E.) of four different determinations within the same experiment. When not shown, the standard error is smaller than the symbol.

Figures 2A and 2B are data on the effect of *mdr1* [S]ODNs plus vincristine on HL-60/Vinc cell proliferation Figure 2A and cell survival (B). In (A) cells were treated with *mdr1* sense (❐), scrambled (■) or antisense (❐) [S]ODNs at a total dose of 200 μg/ml fractioned in 4 days (80 μg/ml at day 0 and 40 μg/ml from day 1 to day 3) and then exposed to vincristine 0.01-1 μg/ml) for 72 hours. Vincristine-treated cells (❐) were exposed to VINC alone (same doses and exposure time). Control cells were left untreated. Representative of four different experiments with similar results. Each value is an average ± standard error (S.E.) of four different determinations within the same experiment. In Fig.2B cells were treated with *mdr1* [S]ODNs plus VINC or with VINC alone as described in Fig. 2A. Control cells were left untreated. At the end of the treatments, methylcellulose clonogenic assays were performed. Colonies were scored 10-12 days after plating and the surviving fractions were calculated by dividing the absolute survival of the treated samples by that of the control samples. (▲) VINC; (O) S + VINC; (●) SCR + VINC; (■) AS + VINC. Representative of three different experiments with similar results. Each value is an average ± standard error (S.E.) of three different determinations within the same experiment. When not show, the standard error is smaller than the symbol.

Figures 3A and 3B show data of expression of *mdr1* mRNA (Fig. 3A) and gP-170 protein (Fig. 3B) in HL-60/Vinc cells treated with *mdr1* [S]ODNs. In Fig. 3A, cells were exposed to *mdr1* S or AS [S]ODNs (as reported in Fig. 2 legend), to 0.5 μg/ml vincristine (for 72 hours) and to *mdr1* S or AS [S]ODNs followed by vincristine. Control cells were left untreated. To assess *mdr1* mRNA level, cells were harvested at the end of treatment, and total RNA was isolated and divided into two equal portions and separately amplified by RT-PCR (35 cycles) with *mdr1* and β-actin-specific primers. Lanes are as follows: 1) untreated cells; 2) vincristine-treated cells; 3) *mdr1* [S]ODNs-treated cells; 4) *mdr1* AS [S]ODNs-treated cells; 5) *mdr1* [S]ODNs

+ vincristine-treated cells; 6) *mdr1* AS [S]ODNs + vincristine-treated cells. Results are from a representative experiment. In (B) cells were untreated (1) or exposed to *mdr1* sense (2), scrambled (3) or antisense (4) [S]ODNs (as described in Fig. 2 legend). gP-170 levels were assessed by Western blot performed at the end of *mdr1* [S]ODNs treatment using an anti-gP-170 MoAb. As a control of protein amounts loaded and blotted, and of [S]ODNs specificity for gP-170 protein down-regulation, HSP 72/73 and MYB protein levels were also evaluated on the same blot. Representative of three different experiments with similar results.

Figures 4A and 4B show survival curves of SCID mice injected with HL-60/Vinc cells and treated with *mdr1* [S]ODNs and vincristine, given alone or in combination (details on the treatment schedules are in the Materials and Methods and Results sections). Fig. 4A shows survival curves relative to the mice of the SCR-treated groups; Fig. 4B shows survival curves of the mice in the AS-treated groups. The survival curves of the untreated and of the vincristine treated-mice are the same in both panels. Each experimental group consisted of 10 mice. The survival curves are as follows: ●—● untreated; ○—○ vincristine; ■—■ scrambled; □—□ scrambled plus vincristine; ▲—▲ antisense; △—△ antisense plus vincristine.

The *P* values obtained from a Mann-Whitney non parametric test comparing all the survival curves among themselves are the following:

Panel A

C versus VINC	*P*=0.15	n.s.
C versus SCR	*P*=0.40	n.s.
C versus SCR+VINC	*P*=0.12	n.s.
VINC versus SCR	*P*=0.4	n.s.
VINC versus SCR+VINC	*P*=0.2	n.s.

Panel B

C versus VINC	*P*=0.15	n.s.
C versus AS	*P*=0.45	n.s.
C versus AS+VINC	*P*=0.005	h.s.
VINC versus AS	*P*=0.15	n.s.
VINC versus AS+VINC	*P*=0.03	s.
AS versus AS+VINC	*P*=0.0045	h.s.

Panel A and B

SCR versus AS	*P*=0.2	n.s.
SCR versus AS+VINC	*P*=0.014	s.
AS versus SCR+VINC	*P*=0.1	n.s.

SCR+VINC versus
AS+VINC P=0.04 s.

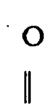
0.01<P values <0.05 - significant (s);
P values <0.01 - highly significant (h.s.);
P values >0.05 - not significant (n.s.).

Definitions

An "antisense oligonucleotide specific for mdr1" or "mdr1 antisense oligonucleotide" is meant an oligonucleotide having a sequence (I) capable of forming a stable triplex with a portion of the mdr1 gene, or (ii) capable of forming a stable duplex with a portion of an mRNA transcript of the mdr1 gene.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including deoxyribonucleosides, ribonucleosides, α-anomeric forms thereof, polyamide nucleic acids, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually, monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g., 3-4, to several hundreds of monomeric units. Analogs of phosphodiester linkages include: phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like, as more fully described below. As used herein, "nucleoside" includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g., as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g., described generally by Scheit, Nucleotide Analogs (John Wiley, New York, 1980). Such analogs include synthetic nucleosides designed to enhance binding properties, e.g., duplex or triplex stability, specificity, or the like.

The term "phosphorothioate oligonucleotide" means an oligonucleotide wherein one or more of the internucleotide linkages is a phosphorothioate group,



$$\begin{array}{c} \sim O - P - O \sim \\ | \\ S^- \end{array}$$

as opposed to the phosphodiester group

$$\begin{array}{c} O \\ \| \\ \sim O - P - O \sim \\ | \\ O^- \end{array}$$

which is characteristic of unmodified oligonucleotides.

By "alkylphosphonate oligonucleoside" is meant an oligonucleotide wherein one or more of the internucleotide linkages is an alkylphosphonate group,

$$\begin{array}{c} O \\ \| \\ \sim O - P - O \sim \\ | \\ R \end{array}$$

wherein R is an alkyl group, preferably methyl or ethyl.

"Stability" in reference to duplex or triplex formation roughly means how tightly an antisense oligonucleotide binds to its intended target sequence; more precisely, it means the free energy of formation of the duplex or triplex under physiological conditions. Melting temperature under a standard set of conditions, e.g., as described below, is a convenient measure of duplex and/or triplex stability. Preferably, antisense oligonucleotides of the invention are selected that have melting temperatures of at least 50°C under the standard conditions set forth below; thus, under physiological conditions and the preferred concentrations, duplex or triplex formation will be substantially favored over the state in which the antisense oligonucleotide and its target are dissociated. It is understood that a stable duplex or triplex may in some embodiments include mismatches between base pairs and/or among base triplets in the case of triplexes. Preferably, antisense oligonucleotides of the invention form perfectly matched duplexes and/or triplexes with their target polynucleotides.

The term "downstream" when used in reference to a direction along a nucleotide sequence means the 5' to 3' direction. Similarly, the term "upstream" means the 3' to 5' direction.

The term "mdr1 mRNA transcript" means the presently known mRNA transcript of the mdr1 gene and all variations thereof, or any further transcripts which may be elucidated.

By "mdr1 gene inactivation" is meant the interruption of expression of functional protein P-170 which is the product of the "mdr1 gene".

As used herein, the term "MDR cancer" is meant to refer to cancer cells which have a multiple drug resistant phenotype as a result of mdr1 gene expression.

As used herein, the term "MDR leukemia" is meant to refer to leukemia cells which have a multiple drug resistant phenotype as a result of mdr1 gene expression.

Detailed Description of the Invention

According to the invention, the MDR phenotype associated with mdr1 gene expression and production of P-170 has been reversed *in vivo* using antisense oligonucleotides to inhibit mdr1 gene expression. The *in vivo* inhibition of P-170 production in MDR cancer cells renders the cancer cells susceptible to chemotherapeutic elimination. Accordingly, the patient can be effectively treated to eliminate the cancer cells upon reversal of the MDR phenotype.

According to the invention, individuals suffering from MDR cancer can treated by administering antisense pharmaceutical compositions which comprise antisense oligonucleotides specific for mdr1 to render the cancer susceptible to drugs. The individual is also administered drugs which eliminate cancer cells.

Those having ordinary skill in the art can readily identify an individual who has cancer which is MDR cancer. Such individuals do not respond to certain chemotherapies and the mdr1 gene expression can be detected in cancer cells such as by detecting overexpression of the mdr1 gene or overproduction of P-170 protein. In some preferred embodiments, the MDR cancer is a blood borne cancer such as MDR leukemia including chronic MDR leukemia and acute MDR leukemia, MDR lymphoma, or MDR myeloma (cancer of plasma cells).

According to the invention, the patient is administered the pharmaceutical composition that comprises the antisense oligonucleotides for at least 7 days, preferably for at least 7-14 days, more preferably 14-21 days or longer. In some preferred embodiments, the antisense oligonucleotides are administered prior to and/or throughout

anti-cancer chemotherapy. Essentially, the antisense oligonucleotide is administered at least 4-7 prior to and preferably throughout a chemotherapeutic cycle. Thus, if a chemotherapeutic cycle is three weeks (from Day 1 to Day 21) including weekly administration of anticancer drugs (on, for example, Day 1, Day 8 and Day 15), the antisense oligonucleotides are administered starting on day -7 to -4 and preferably continuing until at least Day 15-21.

According to the invention, the patient is administered up to 1 gram/day of antisense oligonucleotide. Dosage varies depending upon the physical chemical characteristics of the antisense oligonucleotides, taking into account half life, activity and toxicity. Generally 10 mg to 1 gram are administered. In some embodiments, 70-700 mg of antisense oligonucleotide are administered per day. In some embodiments, 250-500 mg of antisense oligonucleotide are administered per day. In some embodiments, 10-100 mg of antisense oligonucleotide are administered per day.

Modes of administration include intravenous, intraperitoneal or subcutaneous. Administration is preferably continuous as opposed to bolus. According to some preferred embodiments, antisense oligonucleotides are administered with a continuous infusion pump.

According to some preferred embodiments, antisense oligonucleotides are [S]Oligonucleotides, i.e. phosphorothioates.

Anti-cancer drugs may be co-administered to the patient starting on day 1 or administration of such chemotherapeutics may be initiated on any day thereafter. In some preferred embodiments, the patient is treated with antisense oligonucleotides for at least 7 days prior to initiation of anti-cancer chemotherapy.

The cDNA nucleotide sequence of the mdr1 gene and predicted amino acid polypeptide are disclosed in Chen, C.J. et al., Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells, Cell 47:381-389, 1986, which is incorporated herein by reference). The translation initiation codon ATG is preceded by a 5'-untranslated region. The termination codon TGA is followed by a 3'-untranslated region, including a polyadenylation sequence at the 3' end.

Whether or not a particular cancer is a MDR cancer may be ascertained by conventional molecular biological techniques, such as analysis of gene expression or

protein production. Such assays to detect gene expression or protein production are well known. Patients may for example be identified by amplification of mdr1 mRNA using standard procedures for amplification of mRNA sequences (reverse transcriptase, polymerase chain reaction; RT-PCR) or by immunoassay using anti-P170 antibodies.

In the practice of the present invention, target mdr1 polynucleotides may be single-stranded or double-stranded DNA or RNA; however, single-stranded DNA or RNA targets are preferred. It is understood that the target to which the mdr1 antisense oligonucleotides of the invention are directed include allelic forms of the mdr1 gene and mRNA. There is substantial guidance in the literature for selecting particular sequences for antisense oligonucleotides given a knowledge of the sequence of the target polynucleotide, e.g., Peyman and Ulmann, Chemical Reviews, 90:543-584, 1990; Crooke, Ann. Rev. Pharmacal. Toxicol., 32:329-376 (1992); and Zamecnik and Stephenson, Proc. Natl. Acad. Sci., 75:280-284 (1974). Preferably, the sequences of mdr1 antisense compounds are selected such that the G-C content is at least 60%. Preferred mRNA targets include the 5' cap site, tRNA primer binding site, the initiation codon site, the mRNA donor splice site, and the mRNA acceptor splice site, e.g., Goodchild et al., U.S. patent 4,806,463.

Where the target polynucleotide comprises the mdr1 mRNA transcript, oligonucleotides complementary to and hybridizable with any portion of the transcript are, in principle, effective for inhibiting translation, and capable of inducing the effects herein described. It is believed that translation is most effectively inhibited by blocking the mRNA at a site at or near the initiation codon. Thus, oligonucleotides complementary to the 5'-region of the mdr1 mRNA transcript are preferred. Oligonucleotides complementary to the mdr1 mRNA, including the initiation codon (the first codon at the 5' end of the translated portion of the mdr1 transcript), or codons adjacent the initiation codon, are preferred.

While antisense oligomers complementary to the 5'-region of the mdr1 transcript are preferred, particularly the region including the initiation codon, it should be appreciated that useful antisense oligomers are not limited to those oligomers complementary to the sequences found in the translated portion of the mRNA transcript, but also includes oligomers complementary to nucleotide sequences contained in, or extending into, the 5'- and 3'-untranslated regions.

According to a preferred embodiment of the invention, the antisense oligonucleotide is complementary to the mRNA transcript beginning with the initiation codon of the transcript and extending downstream thereof for a distance of 50 nucleotides.

Smaller oligomers based upon the 50-mer sequence, in particular, oligomers hybridizable to segments of the mdr1 message containing the initiation codon, may be utilized. In some embodiments, particularly preferred are oligomers containing at least 12 nucleotides, more preferably 12-25 nucleotides, and in some embodiments, most preferably 18, 19 or 20 nucleotides.

Antisense oligonucleotides of the invention may comprise any polymeric compound capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-nucleoside interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like.

Antisense compounds of the invention may also contain pendent groups or moieties, either as part of or separate from the basic repeat unit of the polymer, to enhance specificity, nuclease resistance, delivery, or other property related to efficacy, e.g., cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end-capping" with one or more nuclease-resistant linkage groups such as phosphorothioate, and the like.

For example, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting an alkyl group or alkoxy group for a phosphate oxygen in the internucleotide phosphodiester linkage to form an alkylphosphonate oligonucleoside or alkylphosphotriester oligonucleotide. Non-ionic oligonucleotides such as these are characterized by increased resistance to nuclease hydrolysis and/or increased cellular uptake, while retaining the ability to form stable complexes with complementary nucleic acid sequences. The alkylphosphonates, in particular, are stable to nuclease cleavage and soluble in lipid. The preparation of alkylphosphonate oligonucleosides is disclosed in Tso et al., U.S. patent 4,469,863.

Preferably, nuclease resistance is conferred on the antisense compounds of the invention by providing nuclease-resistant internucleosidic linkages. Many such linkages are known in the art, e.g., phosphorothioate: Zon and Geiser, Anti-Cancer Drug Design, 6:539-568 (1991); Stec et al., U.S. patent 5,151,510; Hirschbein, U.S. patent 5,166,387; Bergot, U.S. patent 5,183,885; phosphorodithioates: Marshall et al., Science, 259:1564-1570 (1993); Caruthers and Nielsen, International application PCT/US89/02293;

phosphoramidates, e.g., -OP(=O)(NR1R2)-O- with R1 and R2 hydrogen or C1-C3 alkyl; Jager et al., Biochemistry, 27:7237-7246 (1988); Froehler et al., International application PCT/US90/03138; peptide nucleic acids: Nielsen et al., Anti-Cancer Drug Design, 8: 53-63 (1993), International application PCT/EP92/01220; methylphosphonates: Miller et al., U.S. patent 4,507,433, Ts'o et al., U.S. patent 4,469,863; Miller et al., U.S. patent 4,757,055; and P-chiral linkages of various types, especially phosphorothioates, Stec et al., European patent application 506,242 (1992) and Lesnikowski, Bioorganic Chemistry, 21:127-155 (1993). Additional nuclease linkages include phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, alkylphosphotriester such as methyl- and ethylphosphotriester, carbonate such as carboxymethyl ester, carbamate, morpholino carbamate, 3'-thioformacetal, silyl such as dialkyl(C_1-C_6)- or diphenylsilyl, sulfamate ester, and the like. Such linkages and methods for introducing them into oligonucleotides are described in many references, e.g., reviewed generally by Peyman and Ulmann, Chemical Reviews 90:543-584 (1990); Milligan et al., J. Med. Chem., 36:1923-1937 (1993); Matteucci et al., International application PCT/US91/06855.

Resistance to nuclease digestion may also be achieved by modifying the internucleotide linkage at both the 5' and 3' termini with phosphoroamidites according to the procedure of Dagle et al., Nucl. Acids Res. 18, 4751-4757 (1990).

Preferably, phosphorus analogs of the phosphodiester linkage are employed in the compounds of the invention, such as phosphorothioate, phosphorodithioate, phosphoramidate, or methylphosphonate. More preferably, phosphorothioate is employed as the nuclease resistant linkage.

Phosphorothioate oligonucleotides contain a sulfur-for-oxygen substitution in the internucleotide phosphodiester bond. Phosphorothioate oligonucleotides combine the properties of effective hybridization for duplex formation with substantial nuclease resistance, while retaining the water solubility of a charged phosphate analogue. The charge is believed to confer the property of cellular uptake via a receptor (Loke et al., Proc. Natl. Acad. Sci., 86, 3474-3478 (1989)).

It is understood that in addition to the preferred linkage groups, compounds of the invention may comprise additional modifications, e.g., boronated bases, Spielvogel et al., 5,130,302; cholesterol moieties, Shea et al., Nucleic Acids Research, 18:3777-3783

(1990) or Letsinger et al., Proc. Natl. Acad. Sci., 86:6553-6556 (1989); and 5-propynyl modification of pyrimidines, Froehler et al., Tetrahedron Lett., 33:5307-5310 (1992).

Preferably, antisense compounds of the invention are synthesized by conventional means on commercially available automated DNA synthesizers, e.g., an Applied Biosystems (Foster City, CA) model 380B, 392 or 394 DNA/RNA synthesizer. Preferably, phosphoramidite chemistry is employed, e.g., as disclosed in the following references: Beaucage and Iyer, Tetrahedron, 48:2223-2311 (1992); Molko et al., U.S. patent 4,980,460; Koster et al., U.S. patent 4,725,677; Caruthers et al., U.S. patents 4,415,732; 4,458,066; and 4,973,679.

In embodiments where triplex formation is desired, there are constraints on the selection of target sequences. Generally, third strand association via Hoogsteen type of binding is most stable along homo- pyrimidine-homopurine tracks in a double stranded target. Usually, base triplets form in T-A*T or C-G*C motifs (where "-" indicates Watson-Crick pairing and "*" indicates Hoogsteen type of binding); however, other motifs are also possible. For example, Hoogsteen base pairing permits parallel and antiparallel orientations between the third strand (the Hoogsteen strand) and the purine-rich strand of the duplex to which the third strand binds, depending on conditions and the composition of the strands. There is extensive guidance in the literature for selecting appropriate sequences, orientation, conditions, nucleoside type (e.g., whether ribose or deoxyribose nucleosides are employed), base modifications (e.g., methylated cytosine, and the like) in order to maximize, or otherwise regulate, triplex stability as desired in particular embodiments, e.g., Roberts et al., Proc. Natl. Acad. Sci., 88:9397-9401 (1991); Roberts et al., Science, 258:1463-1466 (1992); Distefano et al., Proc. Natl. Acad. Sci., 90:1179-1183 (1993); Mergny et al., Biochemistry, 30:9791-9798 (1992); Cheng et al., J. Am. Chem. Soc., 114:4465-4474 (1992); Beal and Dervan, Nucleic Acids Research, 20:2773-2776 (1992); Beal and Dervan, J. Am. Chem. Soc., 114:4976-4982; Giovannangeli et al., Proc. Natl. Acad. Sci., 89:8631-8635 (1992); Moser and Dervan, Science, 238:645-650 (1987); McShan et al., J. Biol. Chem., 267: 5712-5721 (1992); Yoon et al., Proc. Natl. Acad. Sci., 89:3840-3844 (1992); and Blume et al., Nucleic Acids Research, 20:1777-1784 (1992).

The length of the oligonucleotide moieties is sufficiently large to ensure that specific binding will take place only at the desired target polynucleotide and not at other fortuitous sites, as explained in many references, e.g., Rosenberg et al., International

application PCT/US92/05305; or Szostak et al., Meth. Enzymol, 68:419-429 (1979). The upper range of the length is determined by several factors, including the inconvenience and expense of synthesizing and purifying oligomers greater than about 30-40 nucleotides in length, the greater tolerance of longer oligonucleotides for mismatches than shorter oligonucleotides, whether modifications to enhance binding or specificity are present, whether duplex or triplex binding is desired, and the like. Usually, antisense compounds of the invention have lengths in the range of about 12 to 60 nucleotides. More preferably, antisense compounds of the invention have lengths in the range of about 15 to 40 nucleotides; and most preferably, they have lengths in the range of about 18 to 30 nucleotides.

In general, the antisense oligonucleotides used in the practice of the present invention will have a sequence which is completely complementary to a selected portion of the target polynucleotide. Absolute complementarity is not however required, particularly in larger oligomers. Thus, reference herein to a "nucleotide sequence complementary to" a target polynucleotide does not necessarily mean a sequence having 100% complementarity with the target segment. In general, any oligonucleotide having sufficient complementarity to form a stable duplex with the target (e.g. the mdr1 mRNA) that is, an oligonucleotide which is "hybridizable", is suitable. Stable duplex formation depends on the sequence and length of the hybridizing oligonucleotide and the degree of complementarity with the target polynucleotide. Generally, the larger the hybridizing oligomer, the more mismatches may be tolerated. More than one mismatch probably will not be tolerated for antisense oligomers of less than about 21 nucleotides. One skilled in the art may readily determine the degree of mismatching which may be tolerated between any given antisense oligomer and the target sequence, based upon the melting point, and therefore the thermal stability, of the resulting duplex.

Preferably, the thermal stability of hybrids formed by the antisense oligonucleotides of the invention are determined by way of melting, or strand dissociation, curves. The temperature of fifty percent strand dissociation is taken as the melting temperature, T_m, which, in turn, provides a convenient measure of stability. T_m measurements are typically carried out in a saline solution at neutral pH with target and antisense oligonucleotide concentrations at between about 1.0-2.0 μM. Typical conditions are as follows: 150 mM NaCl and 10mM $MgCl_2$ in a 10 mM sodium phosphate buffer (pH

7.0) or in a 10mM Tris-HCl buffer (pH 7.0). Data for melting curves are accumulated by heating a sample of the antisense oligonucleotide/target polynucleotide complex from room temperature to about 85-90°C. As the temperature of the sample increases, absorbance of 260 nm light is monitored at 1°C intervals, e.g., using a Cary (Australia) model 1E or a Hewlett-Packard (Palo Alto, CA) model HP 8459 UV/VIS spectrophotometer and model HP 89100A temperature controller, or like instruments. Such techniques provide a convenient means for measuring and comparing the binding strengths of antisense oligonucleotides of different lengths and compositions.

Pharmaceutical compositions of the invention include a pharmaceutical carrier that may contain a variety of components that provide a variety of functions, including regulation of drug concentration, regulation of solubility, chemical stabilization, regulation of viscosity, absorption enhancement, regulation of pH, and the like. The pharmaceutical carrier may comprise a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solutions of dextrose, and the like. For water soluble formulations, the pharmaceutical composition preferably includes a buffer such as a phosphate buffer, or other organic acid salt, preferably at a pH of between about 7 and 8. For formulations containing weakly soluble antisense compounds, micro-emulsions may be employed, for example by using a nonionic surfactant such as polysorbate 80 in an amount of 0.04-0.05% (w/v), to increase solubility. Other components may include antioxidants, such as ascorbic acid, hydrophilic polymers, such as, monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, dextrins, chelating agents, such as EDTA, and like components well known to those in the pharmaceutical sciences, e.g., Remington's Pharmaceutical Science, latest edition (Mack Publishing Company, Easton, PA).

Antisense compounds of the invention include the pharmaceutically acceptable salts thereof, including those of alkaline earths, e.g., sodium or magnesium, ammonium or NX4+, wherein X is C1-C4 alkyl. Other pharmaceutically acceptable salts include organic carboxylic acids such as acetic, lactic, tartaric, malic, isethionic, lactobionic, and succinic acids; organic sulfonic acids such as methanesulfonic, ethanesulfonic, and benzenesulfonic; and inorganic acids such as hydrochloric, sulfuric,

phosphoric, and sulfamic acids. Pharmaceutically acceptable salts of a compound having a hydroxyl group include the anion of such compound in combination with a suitable cation such as Na+, NH4+, or the like.

The mdr1 antisense oligonucleotides are preferably administered parenterally, most preferably intravenously. The vehicle is designed accordingly. Alternatively, oligonucleotide may be administered subcutaneously via controlled release dosage forms.

In addition to administration with conventional carriers, the antisense oligonucleotides may be administered by a variety of specialized oligonucleotide delivery techniques. Sustained release systems suitable for use with the pharmaceutical compositions of the invention include semi-permeable polymer matrices in the form of films, microcapsules, or the like, comprising polylactides, copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, poly(2-hydroxyethyl methacrylate), and like materials, e.g., Rosenberg et al., International application PCT/US92/05305.

The oligonucleotides may be encapsulated in liposomes for therapeutic delivery, as described for example in Liposome Technology, Vol. II, Incorporation of Drugs, Proteins, and Genetic Material, CRC Press. The oligonucleotide, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature. The use of liposomes to introduce nucleotides into cells is described in U.S. Pat. Nos. 4,897,355 and 4,394,448, for example. For general methods of preparing liposomes comprising biological materials see, for example, U.S. Pat. Nos. 4,235,871, 4,231,877, 4,224,179, 4,753,788, 4,673,657, 4,247,411 and 4,814,270.

The oligonucleotides may be conjugated to poly(L-lysine) to increase cell penetration. Such conjugates are described by Lemaitre et al., Proc. Natl. Acad. Sci. USA, 84, 648-652 (1987). The procedure requires that the 3'-terminal nucleotide be a ribonucleotide. The resulting aldehyde groups are then randomly coupled to the epsilon-amino groups of lysine residues of poly(L-lysine) by Schiff base formation, and

then reduced with sodium cyanoborohydride. This procedure converts the 3'-terminal ribose ring into a morpholine structure antisense oligomers.

Antisense compounds of the invention also include conjugates of such oligonucleotides with appropriate ligand-binding molecules. The oligonucleotides may be conjugated for therapeutic administration to ligand-binding molecules which recognize cell-surface molecules, such as according to International Patent Application WO 91/04753. The ligand-binding molecule may comprise, for example, an antibody against a cell surface antigen, an antibody against a cell surface receptor, a growth factor having a corresponding cell surface receptor, an antibody to such a growth factor, or an antibody which recognizes a complex of a growth factor and its receptor. Methods for conjugating ligand-binding molecules to oligonucleotides are detailed in WO 91/04753.

In particular, the growth factor to which the antisense oligonucleotide may be conjugated, may comprise transferrin or folate. Transferrin-polylysine-oligonucleotide complexes or folate-polylysine-oligonucleotide complexes may be prepared for uptake by cells expressing high levels of transferrin or folate receptor. The preparation of transferrin complexes as carriers of oligonucleotide uptake into cells is described by Wagner et al., Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990). Inhibition of leukemia cell proliferation by transferrin receptor-mediated uptake of c-myb antisense oligonucleotides conjugated to transferrin has been demonstrated by Citro et al., Proc. Natl. Acad. Sci. USA., 89, 7031-7035 (1992). Cellular delivery of folate-macromolecule conjugates via folate receptor endocytosis, including delivery of an antisense oligonucleotide, is described by Low et al., U.S. Patent 5,108,921. Also see, Leamon et al., Proc. Natl. Acad. Sci. 88, 5572 (1991).

A preferred method of administration of oligonucleotide comprises either systemic or regional perfusion, as is appropriate. According to a method of regional perfusion, the afferent and efferent vessels supplying the extremity containing the lesion are isolated and connected to a low-flow perfusion pump in continuity with an oxygenator and a heat exchanger. The iliac vessels may be used for perfusion of the lower extremity. The axillary vessels are cannulated high in the axilla for upper extremity lesions. Oligonucleotide is added to the perfusion circuit, and the perfusion is continued for an appropriate time period, e.g., one hour. Perfusion rates of from 100 to 150 ml/minute may be employed for lower extremity lesions, while half that rate should be employed for upper

extremity lesions. Systemic heparinization may be used throughout the perfusion, and reversed after the perfusion is complete. This isolation perfusion technique permits administration of higher doses of chemotherapeutic agent than would otherwise be tolerated upon infusion into the arterial or venous systemic circulation.

For systemic infusion, the oligonucleotides are preferably delivered via a central venous catheter, which is connected to an appropriate continuous infusion device. Indwelling catheters provide long term access to the intravenous circulation for frequent administration of drugs over extended time periods. They are generally surgically inserted into the external cephalic or internal jugular vein under general or local anesthesia. The subclavian vein is another common site of catheterization. The infuser pump may be external, or may form part of an entirely implantable central venous system such as the INFUSAPORT system available from Infusaid Corp., Norwood, MA and the PORT-A-CATH system available from Pharmacia Laboratories, Piscataway, NJ. These devices are implanted into a subcutaneous pocket under local anesthesia. A catheter, connected to the pump injection port, is threaded through the subclavian vein to the superior vena cava. The implant contains a supply of oligonucleotide in a reservoir which may be replenished as needed by injection of additional drug from a hypodermic needle through a self-sealing diaphragm in the reservoir. Completely implantable infusers are preferred, as they are generally well accepted by patients because of the convenience, ease of maintenance and cosmetic advantage of such devices.

As an alternative to treatment with exogenous oligonucleotide, antisense oligonucleotide synthesis may be induced in situ by local treatment of the targeted cells with a vector containing an artificially-constructed gene comprising a transcriptional promotor and mdr1 DNA in inverted orientation. The mdr1 DNA for insertion into the artificial gene in inverted orientation comprises cDNA which may be prepared, for example, by reverse transcriptase polymerase chain reaction from RNA using primers derived from the published mdr1 cDNA sequence. Upon transcription, the inverted mdr1 gene segment, which is complementary to the mdr1 mRNA, is produced in situ in the targeted cell. The endogenously produced RNA hybridizes to mdr1 mRNA, resulting in interference with mdr1 function and inhibition of the proliferation of the targeted cell.

The promotor segment of the artificially-constructed gene serves as a signal conferring expression of the inverted mdr1 sequence which lies downstream thereof. It

will include all of the signals necessary for initiating transcription of the sequence. The promotor may be of any origin as long as it specifies a rate of transcription which will produce sufficient antisense mRNA to inhibit the expression of the mdr1 gene, and therefore the proliferation of the targeted cells. Preferably, a highly efficient promotor such as a viral promotor is employed. Other sources of potent promoters include cellular genes that are expressed at high levels. The promotor segment may comprise a constitutive or a regulatable promotor.

The artificial gene may be introduced by any of the methods described in U.S. Patent 4,740,463, incorporated herein by reference. One technique is transfection, which can be done by several different methods. One method of transfection involves the addition of DEAE-dextran to increase the uptake of the naked DNA molecules by a recipient cell. See McCutchin, J.H. and Pagano, J.S., J. Natl. Cancer Inst. 41, 351-7 (1968). Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of Ca^{++} to a phosphate-containing DNA solution. The resulting precipitate apparently includes DNA in association with calcium phosphate crystals. These crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfectant, as well as in its clonal descendants. See Graham, F.L. and van der Eb, A.J., Virology 52, 456-467 (1973) and Virology 54, 536-539 (1973).

Transfection may also be carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). See Felgner et al., Proc. Natl. Acad. Sci., 84, 7413-7417 (1987) (DNA-transfection); Malone et al., Proc. Natl. Acad. Sci., 86, 6077-6081 (1989) (RNA-transfection).

Alternatively, the artificially-constructed gene can be introduced in to cells, in vitro or in vivo, via a transducing viral vector. See Tabin et al., Mol. Cel. Biol. 2, 426-436 (1982). Use of a retrovirus, for example, will infect a variety of cells and cause the artificial gene to be inserted into the genome of infected cells. Such infection could either be accomplished with the aid of a helper retrovirus, which would allow the virus to spread through the organism, or the antisense retrovirus could be produced in a helper-free system, such as Ψ 2-like cells (See Mann et al., Cell 33, 153-160, 1983) that package

amphotropic viruses. A helper-free virus might be employed to minimize spread throughout the organism. Viral vectors in addition to retroviruses can also be employed, such as papovaviruses, SV40-like viruses, or papilloma viruses. The use of retroviruses for gene transfer has been reviewed by Eglitis and Anderson, BioTechniques 6, 608-614 (1988).

Vesicle fusion could also be employed to deliver the artificial gene. Vesicle fusion may be physically targeted to the malignant cells if the vesicle were approximately designed to be taken up by those cells. Such a delivery system would be expected to have a lower efficiency of integration and expression of the artificial gene delivered, but would have a higher specificity than a retroviral vector. A combination strategy of targeted vesicles containing papilloma virus or retrovirus DNA molecules might provide a method for increasing the efficiency of expression of targeted molecules.

Particulate systems and polymers for in vitro and in vivo delivery of polynucleotides were extensively reviewed by Felgner in Advanced Drug Delivery Reviews 5, 163-187 (1990). Techniques for direct delivery of purified genes in vivo, without the use of retroviruses, has been reviewed by Felgner in Nature 349, 351-352 (1991). Such methods of direct delivery of polynucleotides may be utilized for local delivery of either exogenous mdr1 antisense oligonucleotide or artificially-constructed genes producing mdr1 antisense oligonucleotide in situ.

Recently, Wolf et al. demonstrated that direct injection of non-replicating gene sequences in a non-viral vehicle is possible. See Science, 247, 1465-1468 (1990). DNA injected directly into mouse muscle did not integrate into the host genome, and plasmid essentially identical to the starting material was recovered from the muscle months after injection. Interestingly, no special delivery system is required. Simple saline or sucrose solutions are sufficient to delivery DNA and RNA.

For systemic or regional in vivo administration, the amount of antisense oligonucleotide may vary depending on the nature and extent of the neoplasm, the particular oligonucleotide utilized, and other factors. The actual dosage administered may take into account the size and weight of the patient, whether the nature of the treatment is therapeutic in nature, the age, health and sex of the patient, the route of administration, whether the treatment is regional or systemic, and other factors. Intercellular concentrations of from about 1 to about 200 μg/ml at the target polynucleotide may be

employed, preferably from about 10 μg/ml to about 100 μg/ml. The patient should receive a sufficient daily dosage of antisense oligonucleotide to achieve these intercellular concentrations of drug. The daily dosage may range from about 0.2 mg, more preferably from about 25 mg, to about 2 grams per day, with at least about 250 mg per day being preferred. An effective human continuous intravenous infusion dosage, based upon animal studies and Phase I clinical trials employing antisense oligonucleotides targeting other genes in antileukemic therapy, is about 250 mg/day. Greater or lesser amounts of oligonucleotide may be administered, as required. Those skilled in the art should be readily able to derive appropriate dosages and schedules of administration to suit the specific circumstance and needs of the patient. It is believed that a course of treatment may advantageously comprise infusion of the recommended daily dose as a continuous intravenous infusion over 7 days. The oligonucleotide may be given for a period of from about 3 to about 28 days, more preferably from about 7 to about 10 days. Those skilled in the art should readily be able to determine the optimal dosage in each case. For modified oligonucleotides, such as phosphorothioate oligonucleotides, which have a half life of from 24 to 48 hours, the treatment regimen may comprise dosing on alternate days.

For anticancer therapy, the effectiveness of the treatment may be assessed by routine methods which are used for determining whether or not remission has occurred. Such methods generally depend upon some combination of morphological, cytochemical, cytogenetic, immunologic and molecular analyses. In addition, remission can be assessed genetically by probing the level of expression of one or more relevant oncogenes. The reverse transcriptase polymerase chain reaction methodology can be used to detect even very low numbers of mRNA transcript. For example, RT-PCR has been used to detect and genotype the three known bcr-abl fusion sequences in Ph1 leukemias. See PCT/US92/05035 and Kawasaki et al., Proc. Natl. Acad. Sci. USA 85, 5698-5702 (1988).

Typically, therapeutic success is assessed by the decrease and the extent of the primary and any metastatic diseases lesions. For solid tumors, decreasing tumor size is the primary indicia of successful treatment. Neighboring tissues should be biopsied to determine the extent to which metastasis has occurred. Tissue biopsy methods are known to those skilled in the art. More recent methods for detecting cancer cells have focused on detecting the presence of the gene for the relevant oncogene, or its corresponding mRNA. See for example the following U.S. Patents: 4,681,840, 4,857,466 and 4,874,853. The

presence of even a few copies of the target oncogene can be effectively detected by amplification using reverse transcriptase polymerase chain reaction technology. For a detailed discussion of such methods, see for example, Cancer: Principles & Practice of Oncology, edited by V. T. DeVita, S. Hellman and S.A. Rosenberg, J.B. Lippincott Company, Philadelphia, PA (3rd ed., 1989). Methods for diagnosing and monitoring the progress of neoplastic disorders vary depending upon the nature of the particular disease.

The present invention may be used ex vivo to reverse MDR in cells removed form the patient which are to be reintroduced. For example, in bone marrow transplant procedures, bone marrow may be removed from the patient, cultured in the presence of antisense oligonucleotides of the invention and then exposed to convential cytostatic compositions prior to return into the patient. The ex vivo culturing of the bone marrow according to the invention will reverse MDR of cancer cells present in the cultured bone marrow.

The practice of the invention is illustrated by the following non-limiting examples.

Example

In this study, *in vitro* and *in vivo* reversion of MDR was attempted in a human leukemia cell line resistant to vincristine (HL-60/Vinc), using an 18-mer *mdrl* antisense phosphorothioate oligodeoxynucleotide ([S]ODN) in combination with vincristine. As control of sequence specificity, both sense and scrambled [S]ODNs were used. The ability of these [S]ODNs to reverse MDR was studied *in vitro* and in SCID mice. *In vitro* treatment with antisense [S]ODNs restored vincristine sensitivity of HL-60/Vinc cells, while no changes in drug sensitivity were observed upon treatment with the sense or scrambled sequence. The *in vitro* effects correlated with inhibition of P-170 expression in HL-60/Vinc cells exposed to the *mdrl* antisense [S]ODNs. *In vivo* reversal of MDR was obtained in SCID mice injected with HL-60/Vinc cells and systemically treated with [S]ODNs plus vincristine, as indicated by a significantly prolonged survival of SCID mice that received the combination therapy of *mdrl* antisense [S]ODNs+vincristine. Treatments with *mdrl* antisense or scrambled [S]ODNs, vincristine, or scrambled [S]ODNs+vincristine had no effect on mice survival. These results suggest that the use of *mdrl* antisense ODNs in combination with standard antineoplastic drugs might be useful to reverse multidrug resistance *in vitro* and *in vivo*.

Material and Methods

Cell line and culture conditions.

The HL-60/Vinc human promyelocitic cell line isolated from parental HL-60 cells for resistance to vincristine (Krishnamachary, N. et al., The MRP gene associated with non-P-glycoprotein Multidrug Resistance encodes a 190-KDa membrane bound glycoprotein, Cancer Res. 53:3658-3661, 1993, which is incorporated herein by reference) was kindly provided by Dr. Melvin Center. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO), 102 μg/ml L-glutamine, in a humid, 5% CO_2 incubator at 37°C. Cells were grown to a density of $1x10^5$ cells/ml before subculturing and were routinely tested for mycoplasma contamination using a commercial kit (Boehringer Mannheim GmbH, Mannheim, Germany). HL-60/Vinc cells were not maintained in the presence of vincristine because *mdrl* is constitutively overexpressed as confirmed by routinely checking *mdrl* mRNA and P-170 glycoprotein levels. However, fresh aliquots of HL-60/ Vinc cells and HL-60 parental cells (as negative control) were defrosted from liquid nitrogen stocks every 2 months during the study, and analyzed for *mdrl* expression and drug-response before use in each experiment.

Drug and oligodeoxynucleotides (ODNs).

Vincristine (VINC, Oncovin, Eli Lilly, Indianapolis, IN), as supplied for clinical use, was dissolved in double distilled water and further diluted in Hank's Balanced Salt solution (HBSS, GIBCO BRL, Grand Island, NY) to obtain the desired final concentrations. VINC stock solutions were made-up freshly before each experiment.

Phosphorothioate oligodeoxynucleotides ([S]ODNs) were synthesized on an Applied Biosystems (Foster City, CA) DNA automated synthesizer model 380B and were kindly provided by Lynx Therapeutics, Inc. (Hayward, CA). The sequences of the *mdrl* antisense and sense [S]ODNs are 5'-GTCCCCTTCAAGATCCAT-3' (SEQ ID NO:1) and 5'ATGGATCTTGAAGGGGAC-3' (SEQ ID NO:2), respectively. They are complementary (or corresponding) to codons 1-6 of the published human *mdrl* cDNA sequence (Chen, C.J. et al., Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells, Cell 47:381-389, 1986, which is incorporated herein by reference). As control of sequence specificity, an 18-mer [S]ODN with base content identical to the antisense sequence but

in "scrambled" order was also used. [S]ODNs were resuspended (10 mg/ml) in sterile HBSS and stored at -20°C in small aliquots.

***In vitro* [S]ODNs and drug treatment.**

To assess whether individually used *mdrl* ODNs had any effect on cell proliferation, $2x10^4$ HL-60/Vinc cells were seeded in 24-well plates (Costar Corp., Cambridge, MA) in complete RPMI 1640 medium and treated with *mdrl* sense, antisense or scrambled [S]ODNs at a total dose of 200 μg/ml over 4 days (80 μg/ ml at day 0 and 40 μg/ml from day 1 to day 3) over a total dose of 360 μg/ml over 8 days (80 μg/ml at day 0 and 40 μg/ml from day 1 to day 7). Control cells were grown in the same conditions without [S]ODNs. Cell counts and viability (trypan blue dye exclusion) were determined daily until the 8th day of culture on quadruplicate samples from each treatment.

To assess the antiproliferative effects of the [S]ODNs, drug combination, $2x10^4$ HL-60/Vinc cells were seeded in 24-well plates and treated with sense, antisense, or scrambled *mdrl* [S]ODNs at a total dose of 200 μg/ml, as described above. [S]ODNs-treated cells were then exposed to VINC, at doses from 0.01 to 1 μg/ml for 72 hours. Control cells were grown in the same conditions without [S]ODNs and drug. At the end of the combination treatment, quadruplicate samples from each group were harvested, counted and assayed for viability (trypan blue dye exclusion).

Colony assay

$2x10^4$ HL-60/Vinc cells, seeded in 24-well plates, were treated with *mdrl* [S]ODNs and VINC as described in the previous section. At the end of treatment, cell suspension aliquots from control and treated groups were plated in semisolid methycellulose medium HCC 4230 (Stem Cell Technologies, Inc., Vancouver, Canada) in triplicate 35-mm Petri dishes. Colony formation was scored on days 10 to 12. The surviving fractions were calculated by dividing the absolute survival of each treated sample by the absolute survival of the control sample.

Western Blotting

$1x10^5$ HL-60/Vinc cell were plated in 6-well plates (Costar) and treated with *mdrl* sense, antisense or scrambled [S]ODNs at a total dose of 200 μg/ml over 4 days as described above. At the end of [S]ODNs treatment, cells were collected, counted, assayed for viability and washed twice with calcium- and magnesium- free cold phosphate buffered saline (PBS). $1x10^6$ cells from each sample were solubilized in cell lysis buffer

(50 mM Hepes pH 7.0, 500 mM NaCl, 1% NP-40, 10 μg/ml each aprotinin and leupeptin, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM $NaVO_4$ 1mM EDTA) kept on ice for 30 minutes, frozen at -80°C and thawed on ice. Cell lysates were centrifuged at 13,600 x g at 4°C for 15 min and postnuclear supernatant were collected. Equal amounts of protein lysates (50 μg) were separated on 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE) and transferred to nitrocellulose (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Richmond, CA) in glycine transfer buffer (192 mM glycine, 25 mM Tris pH 8.8, 20% v/v methanol). Filters were saturated with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.5%, Tween 20 and then incubated (12 h. 4°C) with the C219 anti-P-170 monoclonal antibody (Signet Laboratories, Inc., Dedham, MA) at a concentration or 0.4μg/mi in TBS containing 2% nonfat dry milk and 0.05% Tween 20. Filters were washed 5 times with 0.25% Tween 20/0.25% NP-40 in TBS and incubated with sheep antimouse IgG linked to horseradish peroxidase Amersham Corp., Arlington Heights, IL at 1:10,000 dilution in 2% nonfat dry milk in TBS (1h, room temperature). Bound proteins were detected using the ECL Western Blotting detection system (Amersham Corp.) according to the manufacturer's instructions. To assess protein amounts loaded and transferred onto nitrocellulose membrane, filters were stripped from the P-170 antibody and blotted with murine anti-human heat-shock protein (HSP 72, 73, Oncogene Science Inc., Uniondale, NJ). As further control or [S]ODNs specificity for P-170 protein, after stripping, filters were incubated with an anti-myb monoclonal antibody (UBI 05-175, Lake Placid, NY). Both HSP 72/73 and c-mvb antibody were used at 1:1,000 dilution.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Total RNA was extracted from cells by the acid quanidinium thiocyanate-phenol-chloroform technique (Chomczynski, P. et al., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem., 162:156-159, 1987, which is incorporated herein by reference), *mdr1* and β-actin mRNA transcripts were detected by RT-PCR, *mdr1* expression was detected with 3' and 5' primers corresponding to nucleotides 535-556 and 361-381, respectively, of the published cDNA sequence. Using these primers, PCR yields a 196-base-pair (bp) product (Noonan, K.E. et al., Quantitative analysis of MDR1 (multidrug resistance) gene expression in

human tumors by polymerase chain reaction., Proc. Natl. Acad. Sci. USA, 87:7160-7164, 1990 which is incorporated herein by reference). Evaluation of β-actin expression, used as control of the RNA amount, was carried out by using 3' and 5' primers corresponding to nucleotides 885-905 and 600-621 respectively, which yield a 306-bp product.

RT reaction:

RNA from each sample was divided into two aliquots (0.5 μg each) that were separately reverse-transcribed using 400 U of Moloney murine leukemia virus reverse transcriptase (Mo-MLV-RT, BRL, Gaithersburg, MD) and 100 ng or *mdr1* or β-actin 3' primer for 1 hour at 37°C.

PCR reaction: The resulting cDNA fragments were amplified with 5' U or Taq polymerase (Boehringer Mannheim) and 200 ng of *mdr1* or β-actin and 5' primers in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT) for 35 cycles or sequential denaturation (at 94°C for 45 seconds), annealing (at 55%C for 45 seconds), and extension (at 72°C for 30 seconds). After the last cycle, all PCR products were subjected to a final extension of 10 minutes at 72°C. After PCR reaction, 20 μl of amplification products were electrophoresed on 2% agarose gel, transferred to Zeta-probe membranes (Bio-Rad Laboratories) and hybridized overnight at 49°C using 5'-end-labeled ($\gamma^{[32]}$P-ATP) oligonucleotide probes. β-actin and *mdr1* PCR transcripts were detected with probes corresponding to nucleotides 795-815 and 478-501, respectively. After hybridization, filters were washed in 2xSSC. 0.1%SDS at 50°C and exposed to X-ray films at -80°C.

Mice, leukemia cell inoculation, and in vivo treatment with mdr1 [S]ODNs and vincristine.

SCID (severe combined immunodeficient) mice were purchased from Taconic Farms (Germantown, NY) and maintained under sterile conditions. SCID mice are excellent hosts for human hematopoietic cells, because defective T- and B-lymphocyte development makes these animals severely immunocompromised. Nevertheless, the presence of residual immune cells usually requires that such animals be irradiated prior to engrafting of human hematopoietic tissue. Male SCID mice (6-8 weeks old) were irradiated (200 cGy total body irradiation), and the following day (day 1) injected intravenously (i.v.) with 5 x 10^7 HL-60/Vinc cells in 0.2 mi of RPMI 1640 medium. [S]ODNs (antisense or scrambled) were administered i.v. starting 7 days after leukemic cells inoculation, at a dose of 1 mg/mouse/day for 10 consecutive days (days 8-17).

Vincristine (VINC) treatment was started 10 days after inoculation of leukemic cells, by i.v. injection or 10% of the previously calculated lethal dose (LD_{10}), subdivided into 3 injections of 20 µg/mouse/day (days 11, 14, 17). The following schedules were employed: a) *mdr1* antisense [S]ODNs x 10 days; b) *mdr1* scrambled [S]ODNs x 10 days; c) vincristine x 3 days; d) *mdr1* antisense [S]ODNs x 10 days plus vincristine x 3 days; e) *mdr1* scrambled [S]ODNs x 10 days plus vincristine x 3 days. Each experimental group consisted of 10 mice.

The effect of the different treatments was evaluated in terms of survival of HL-60/Vinc injected mice. Survival times were calculated from the day of leukemic cell injection. Statistical significance of survival differences among animals in the various treatment groups was assessed using the Mann-Whitney non parametric method. P values <0.01 were considered highly significant P values <0.05 were judged to be of statistical significance; P values >0.05 were considered non significant.

Results and Discussion

Effect of *mdr1* [S]ODNs on HL-60/Vinc cell proliferation.

We first assessed the effects of mdr1 [S] ODNs on *in vitro* HL-60/Vinc cell proliferation (Fig. 1A and 1B). As indicated by the growth curves of HL-60/Vinc cells exposed to *mdr1* sense (S) or antisense (AS) [S]ODNs at a total dose of 200 µg/ml over 4 days (Fig. 1A), and by the growth curves or HL-60/Vinc cells treated with mdr1 scrambled (SCR) and AS [S]ODNs at a total dose of 360µg/ml over 8 days (Fig. 1B), neither treatment had an effect on the proliferation of HL-60/Vinc cells. These results clearly indicate that in our experimental conditions, the selected [S]ODNs were non toxic to HL-60/Vinc cells, and could be used in combination experiments with anticancer agent to attempt reversal or multidrug resistance.

In vitro reversal of multidrug resistance in HL-60/Vinc cells treated with *mdr1* AS [S]ODNs and vincristine.

The ability of *mdr1* AS [S]ODNs to increase vincristine cytotoxicity in HL-60/Vinc resistant cells and thus to reverse multidrug resistance was assessed in combination experiments in which HL-60/Vinc cells were first exposed to *mdr1* AS [S]ODNs and then to VINC (see the Materials and Methods section for details) (Fig. 2A). Treatment with vincristine alone had modest effects on HL-60/Vinc cell proliferation; only at the highest dose (1µg/ml) the inhibition of cell proliferation, expressed as percentage of

the untreated control, reach 48%. Of the three combination treatments including mdr1 [S]ODNs and vincristine, only the exposure to AS [S]ODNs followed by vincristine significantly increased VINC cytotoxicity; the inhibition of cell growth was ~58% at the lowest VINC dose used 0.01 μg/ml) and increased up to ~92%, at the highest VINC dose (1 μg/ml). In contrast, no significant effect was observed when vincristine was combined with S or SCR [S]ODNs. The in vitro restoration of vincristine sensitivity in HL-60/Vinc cells was confirmed by the vincristine IC_{50} value (VINC dose that caused 50% of growth inhibition) for each treatment. IC_{50} values were calculated from the percent growth inhibition caused by the different treatments on HL-60/Vinc cells. HL-60 parental cells and HL-60/Vinc cells showed a marked difference in the IC_{50} value following exposure to VINC (0.008 and 0.95 μg/ml, respectively), with a resistance index of about 120. Treatment of HL-60/Vinc cells with mdr1 S or SCR [S]ODNs followed by vincristine did not significantly change the IC_{50} and .85 μg/ml, respectively. However, a strong decrease of the IC_{50} value was observed in HL-60/Vinc cells treated with the combination mdr1 AS [S]ODNs plus VINC in this case the VINC dose able to kill 50% of HL-60/Vinc cells fell to 0.008 μg/ml about a 120-fold decrease). Of note, the IC_{50} value of the resistant cells exposed to AS+VINC was essentially identical to that of HL-60 parental cells exposed to the drug alone, consistent with a complete restoration of the in vitro sensitivity to vincristine in HL-60/Vinc cells.

The HL-60 Vinc human leukemia cell line selected in vitro for resistance to vincristine exhibits cross-resistance to other MDR related drugs commonly used as antineoplastic agents. Accordingly, we combined mdr1 [S]ODNs with adriamycin ADR) and obtained reversal of ADR resistance (percent cell growth inhibition and IC_{50} value decrease) only when HL-60. Vinc cells were exposed to mdr1 AS [S]ODNs followed by ADR.

Figure 2B reports the survival curves from methycellulose colony assays performed on HL-60/Vinc cells treated with mdr1 [S]ODNs and vincristine (see Materials and Methods section). The combinations S+VINC and SCR+VINC did not cause any increase in the lethal effect or VINC since the survival curves of cells treated with VINC alone, or S+VINC or SCR+VINC are superimposable and the surviving fractions have similar values (about 90% at 0.01 μg/ml and about 50% at 1 μg/ml). Only the treatment with the AS-VINC combination enhanced the lethal effect induced by VINC, as indicated

by the decrease of the surviving fractions (60% at 0.01 μg/ml or VINC, and only 7% at the highest VINC dose employed). These results indicate that *mdr1* AS [S]ODNs are effective in sensitizing HL-60/Vinc resistant cells to vincristine, thus reversing MDR *in vitro.*

Expression of *mdr1* mRNA and gP-170 protein levels in mdr1 [S]ODNs-treated HL-60/Vinc cells.

In an attempt to correlate the antiproliferative effect of the various compounds with levels of mdr1 mRNA, we evaluated *mdr1* mRNA expression in HL-60/Vinc cells immediately after treatment with mdr1 [S]ODNs plus vincristine (Fig. 3A). In comparison with untreated cells (lane 1), cells treated with mdr1 AS [S]ODNs (lane 4) and mdr1 AS [S]ODNs plus vincristine (lane 6) exhibited a marked reduction of mdr1 mRNA levels. No decrease in mdr1 mRNA expression was detected after other treatments (lanes 2, 3 and 5). The levels of β-actin mRNA were essentially unchanged in cells receiving the different treatments, confirming the specificity or the effects induced by mdr1 AS [S]ODNs. These experiments were performed only with the vincristine dose of 0.5 μg/ml, because the antiproliferative effect of the highest dose (1 μg/ml) was too marked when used in combination with the antisense [S]ODNs (Fig. 2A).

Due to the long half-life of mdr1 protein and the small number of cells available after exposure to mdr1 AS [S]ODNs plus VINC, we assessed the ability of mdr1 [S]ODNs to down-regulate mdr1 protein levels only in [S]ODNs-treated cells.

As shown in Fig. 3B, there was no difference in gP-170 protein levels in control, S-or SCR-treated cells (lanes 1, 2 and 3, respectively). However, a decrease in gP-170 protein level was detected after exposure of HL-60/Vinc cells to mdr1 AS [S]ODNs (lane 4) at the same dose used to assess the effects on cell proliferation (Fig. 1 and 2). The levels of HSP 72/73 protein and MYB protein did not diminish in AS [S]ODNs-treated cells, suggesting that the down-regulation of P-170 glycoprotein expression was a specific effect of the mar1 AS [S]ODNs treatment.

Effects of mdr1 [S]ODNs and vincristine on survival of SCID mice injected with HL-60/Vinc cells. To assess the ability of mdr1 [S]ODNs given in combination with vincristine to reverse MDR in vivo, SCID mice injected with HL-60/VINC leukemia cells were treated, by tail vein injection, with mar1 SCR or AS [S]ODNs for 10 days (1 mg/mouse/day) in combination with vincristine for 3 days (20 μg/mouse/day) during

[S]ODNs administration. The control groups received SCR [S]ODNs, AS [S]ODNs, vincristine, or no treatment. Figures 4A and 4B reports the survival curves of HL-60 Vinc SCID mice treated with [S]ODNs and vincristine, individually or in combination. To better distinguish the different survival curves, the curves relative to the mice in the SCR-treated groups (Fig. 4A) were separated from those of the mice of the AS-treated groups (Fig. 4B). For a better comparison of the different treatments, the survival curves of the untreated and the vincristine-treated mice are the same in each panel. The administration of vincristine alone did not prolong the survival of HL-60/Vinc SCID mice, consistent with the in vitro resistance of the leukemic cells to the drug. The untreated mice were all dead 84 days after implant of HL-60/Vinc cells, while the mice in the vincristine-treated group were all dead 91 days after leukemia inoculation. The median survival times were 56 and 70 days for the control and the vincristine group, respectively, such difference was not statistically significant). As expected, the treatment with SCR [S]ODNs alone or SCR [S]ODNs plus vincristine did not affect SCID mice survival, both curves being very similar to that of the control mice (untreated and vincristine-treated). The mice in the SCR-treated group were all dead within 85 days, and those in the SCR plus vincristine-treated group within 92 days from leukemic cells inoculation, with a median survival time of 71 and 77 days, respectively. The treatment with mdr1 AS [S]ODNs was not able by itself to affect SCID mice survival (Fig. 4B). The mice were all dead 100 days after leukemic cells injection, with a median survival time of 57 days (the median survival time of untreated mice was 56 days). The statistical analysis of the control versus the AS curve gave a non-significant P value (0.45) indicating that the observed delay in the death of the antisense-treated mice compared with controls (about 20 days) was only apparent. These results confirm those obtained in vitro indicating that HL-60/Vinc cell growth was not affected by mdr1. As [S]ODNs by themselves both at low (Fig. 1A) and at high doses (Fig. 1B). No significant differences were observed by comparing the survival curve of the AS-treated mice with that of mice with vincristine alone (median survival time 57 and 70 days, respectively, and P value not statistically significant). In contrast, the survival curve of the mice treated with the combination mdr1 AS [S]ODNs plus vincristine is completely different from the others with 60% of the animals in this group still alive 300 days after leukemic cells injection. The median survival time of the vincristine-treated mice was then 300 days, and the statistical analysis gave P values all included in the

significant and in the highly significant range with each of the comparisons: C vs AS+VINC = 0.005; AS vs AS-VINC = 0.0045; VINC vs AS+VINC = 0.03; AS + VINC vs SCR = .014; AS+VINC vs SCR+VINC = 0.04.

300 days after leukemia cell injection, surviving mice from the AS plus vincristine-treated group were sacrificed. Histopathological examination of hematopoietic and non-hematopoietic organs, as well as RT-PCR analysis of c-myb transcripts used as a marker of leukemic cell load did not reveal the presence of tumor cells at a detectable level. Thus, our in vivo results indicate that we have restored vincristine sensitivity in HL-60/Vinc resistant cells injected in SCID mice by treatment with mar1 AS [S]ODNs in combination with vincristine.

The development of multidrug resistance (MDR) by cancer cells represents one of the major reasons of anticancer chemotherapy failure. The present invention modulated and reversed multidrug resistance in a human leukemia resistant cell line, both in vitro and in vivo, using AS [S]ONDs targeted to the mdr1 mRNA. HL-60/Vinc cells that were selected from HL-60 parental cells for resistance to vincristine overexpressed the p-170KDa mdr1 gene product and exhibited cross-referenced to other antineoplastic drugs related to the MDR phenotype, most likely as consequence or increased transport or anticancer agents out of cells and/or decreased intracellular accumulation.

Using an 18 mer antisense [S]ODN targeting the region immediately downstream from the translation initiation codon we observed a marked, but not complete down-regulation of gP-170 protein, consistent with its half-life of about 72 hours. Nevertheless, the observed reduction in gP-170 level was sufficient to block its drug efflux effect, as indicated by the complete restoration of the in vitro sensitivity to vincristine (Fig. 2A, Table 1), upon treatment of HL-60/Vinc cells with mar1 AS [S]ODNs and vincristine. On the other hand, the down-regulation of mdr1 mRNA induced by the AS[S]ODNs was more evident and almost complete (Fig. 3A), suggesting that a 4-day continuous exposure to the AS [S]ODNs was sufficient to obtain a strong inhibition of its expression, even if the mdr1 mRNA is abundant and has a relatively long half-life of 4-5 hours.

The observed in vitro reversal of multidrug resistance correlated well with the results obtained in vivo using SCID mice injected with the drug resistant leukemic cells. In vivo, mdr1 AS [S]ODNs given in combination with vincristine were able to

sensitize the resistant leukemic cells to the effect of vincristine and thus to reverse MDR and prolong survival of leukemic mice. The prolonged survival of leukemic mice by means or sequence-specific [S]ODNs in combination with vincristine supports a specific mechanism of action of mdr1 antisense [S]ODNs in vivo. However, [S]ODNs also exhibit sequence-independent effects that may reflect the ability of such compounds to interact with cellular and extracellular proteins. Thus, we cannot exclude the possibility that an apparently sequence-specific effect is indeed sequence-independent, or that sequence-independent and sequence-specific effects coexist. In conclusion, our studies indicate that [S]ODNs, or newly analogues thereof, targeted to the mdr1 mRNA reverse multidrug resistance in vivo.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

All references cited herein with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(I) APPLICANT: Calabretta, Bruno
(ii) TITLE OF INVENTION: COMPOSITIONS FOR AND METHODS OF TREATING MULTIPLE DRUG RESISTANCE
(iii) NUMBER OF SEQUENCES: 2
(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEES: Woodcock Washburn Kurtz Mackiewicz & Norris.
(B) STREET: One Liberty Place, 46th floor
(C) CITY: Philadelphia
(D) STATE: Pennsylvania
(E) COUNTRY: U.S.A.
(F) ZIP: 19103
(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.50 inch
(B) COMPUTER: IBM compatible
(C) OPERATING SYSTEM: Windows 3.1
(D) SOFTWARE: WordPerfect 6.0
(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/026,958
(B) FILING DATE: 24-SEP-1996
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: DeLuca, Mark
(B) REGISTRATION NUMBER: 33,229
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(B) TELEFAX: (215) 568-3439

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(xi)SEQUENCE DESCRIPTION: SEQ ID NO:1:
GTCCCCTTCA AGATCCAT 18
(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 Nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
ATGGATCTTG AAGGGGAC 18

CLAIMS

1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an antisense oligonucleotide specific for mdr1.

2. A composition according to claim 1 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

3. A composition according to claim 1 wherein the oligonucleotide is an oligodeoxynucleotide.

4. A composition according to claim 1 wherein the oligonucleotide is capable of forming a stable duplex with a portion of an mdr1 mRNA transcript.

5. A composition according to claim 4 wherein the oligonucleotide is capable of forming a stable duplex with a portion of an mdr1 mRNA transcript lying within about 50 nucleotides of the translation initiation codon.

6. A composition according to claim 4 wherein the oligonucleotide comprises from a 12-mer to a 50-mer.

7. A composition according to claim 6 wherein the oligonucleotide comprises from a 15-mer to a 40-mer.

8. An antisense oligonucleotide specific for mdr1 having a length of from about 8 to about 50 nucleotides which is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

9. An oligonucleotide according to claim 8 which is capable of forming a stable duplex with a portion of an mdr1 mRNA transcript.

10. An oligonucleotide according to claim 9 which is capable of forming a stable duplex with a portion of an mdl1 mRNA transcript lying within about 50 nucleotides of the translation initiation codon.

11. An oligonucleotide according to claim 10 having a length of from about 15 to about 40 nucleotides.

12. An oligonucleotide according to claim 11 having a length of from about 18 to about 30 nucleotides.

13. A method for treating an individual who has cancer with cells that are multiple drug resistant due to mdl1 gene expression, the method comprising the steps of:

administering an amount of an antisense oligonucleotide specific for mdr1 sufficient to inhibit mdr1 gene expression for at least 4 days, and

administering an amount of an anticancer drug sufficient to kill cancer cells in which mdr1 expression has been inhibited.

14. The method of claim 13 wherein said individual has leukemia.

15. The method of claim 14 wherein said individual is administered 70-700 mg/day antisense oligonucleotide specific for mdr1.

16. The method of claim 14 wherein said individual is administered 70-700 mg/day antisense oligonucleotide specific for mdr1 for at least 7 days.

17. The method of claim 16 wherein said individual is administered said antisense oligonucleotide specific for mdr1 intravenously, intraperitoneally or subcutaneously by continuous infusion pump.

18. The method of claim 15 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

19. The method of claim 13 wherein said anticancer drug is vinblastine and vincristine, adriamycin, taxol, actinomycin D or mitomycin.

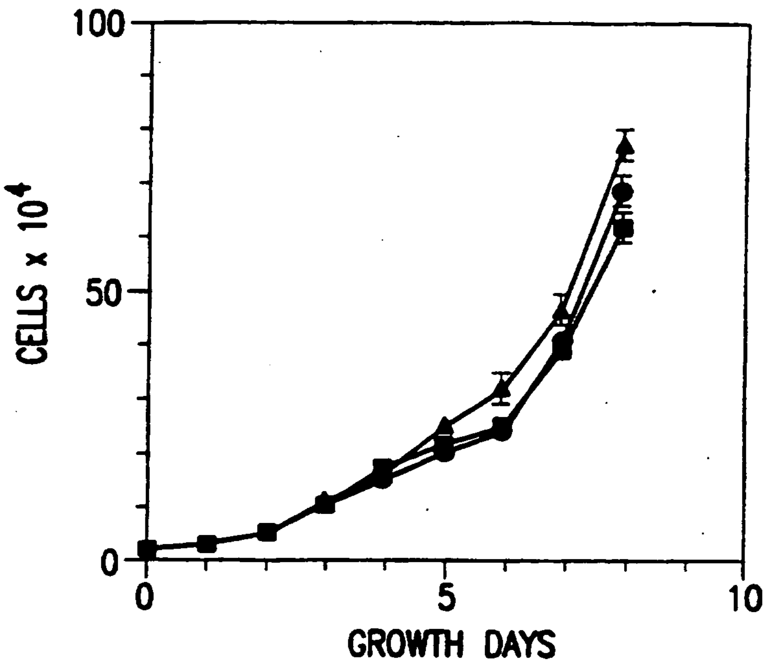
20. The method of claim 13 wherein:

said individual has leukemia,

said oligonucleotide is a phosphorothioate oligonucleotide, and

said individual is administered 70-700 mg/day antisense oligonucleotide specific for mdr1 for at least 7 days intravenously, intraperitoneally or subcutaneously by continuous infusion pump

21. The method of claim 20 wherein said anticancer drug is administered vinblastine and vincristine, adriamycin, taxol, actinomycin D or mitomycin.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0
5
10
GROWTH DAYS

FIG.1A

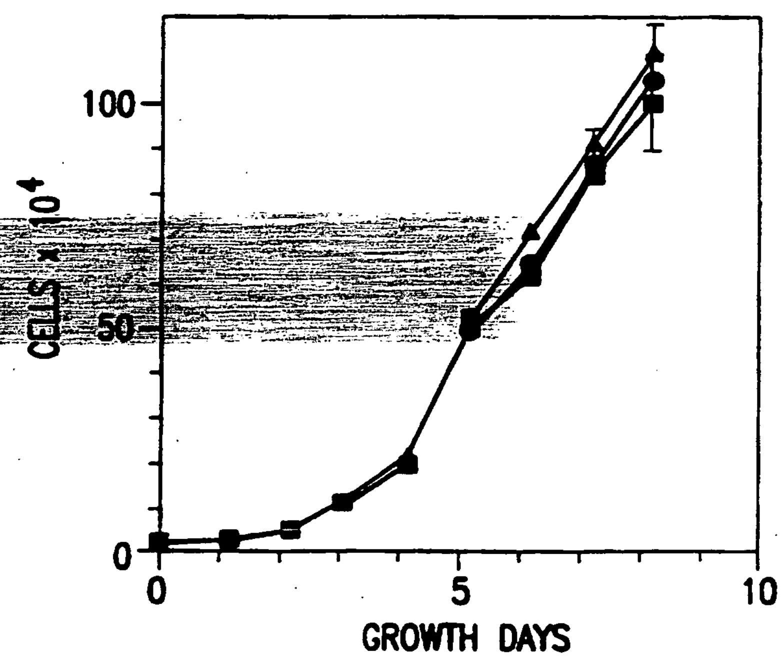
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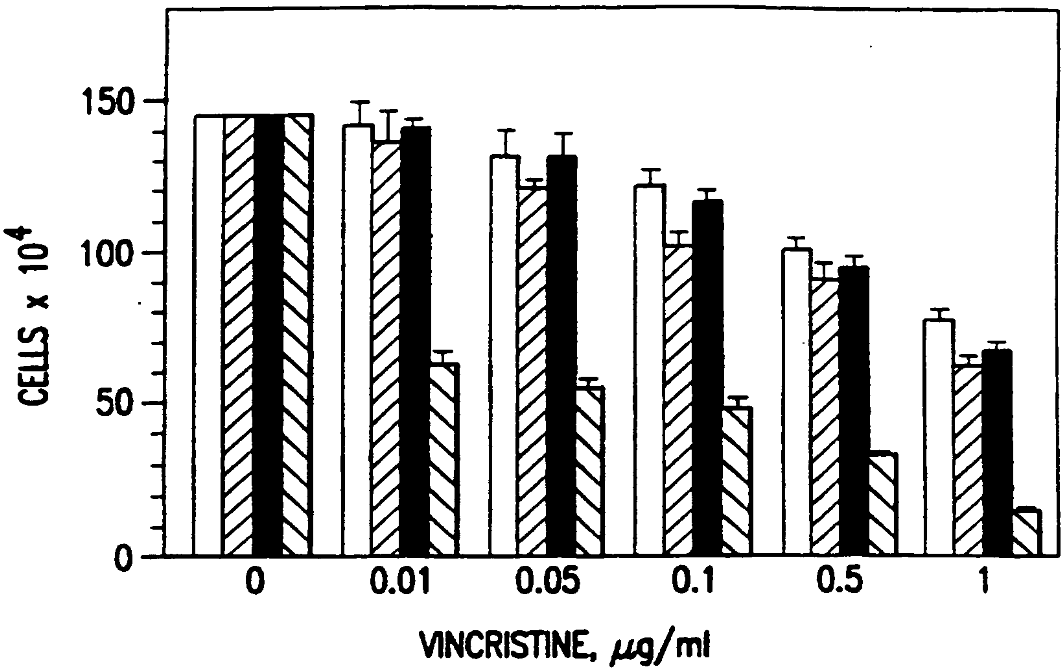
FIG.1B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50
0
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0.05
0.1
0.5
1
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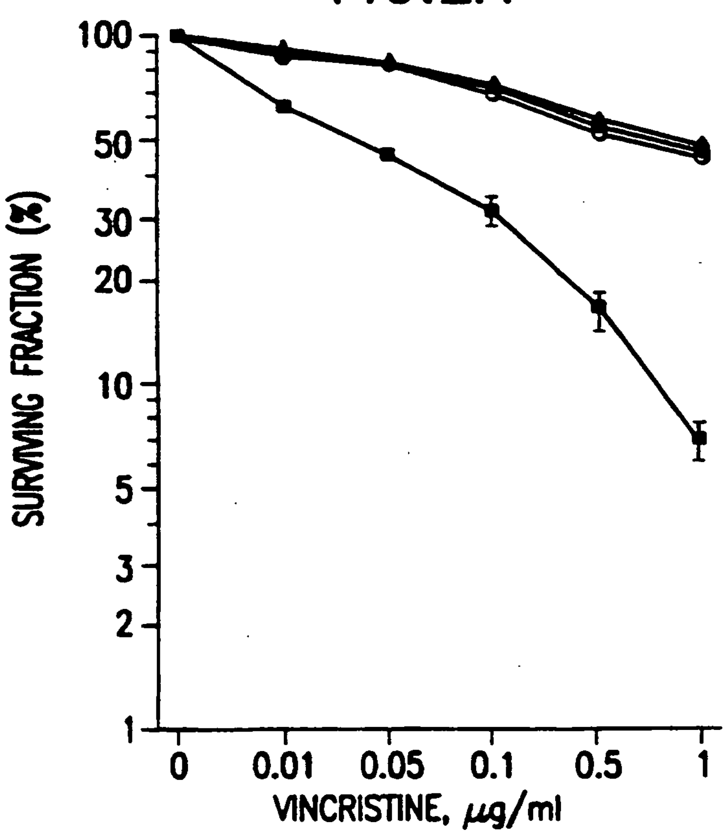
FIG.2A


100
50
30
20
10
5
3
2
1
SURVIVING FRACTION (%)
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0.01
0.05
0.1
0.5
1
VINCRISTINE, μg/ml

FIG.2B

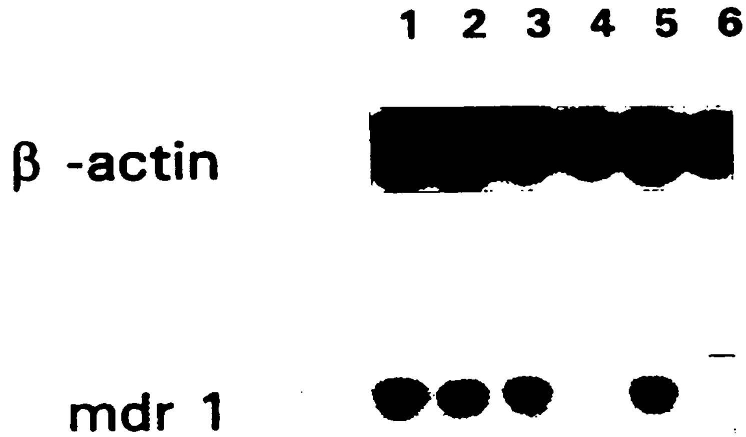


FIG.3A

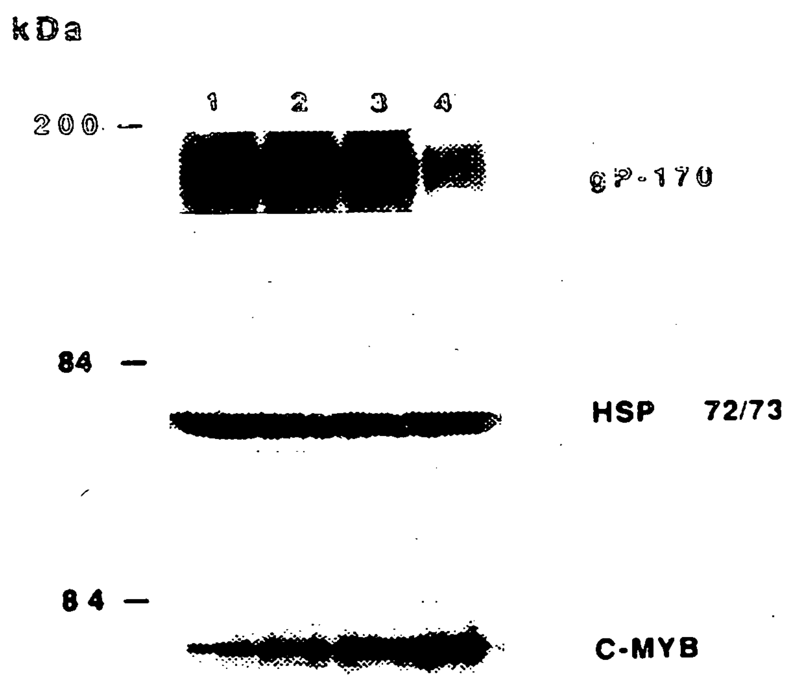
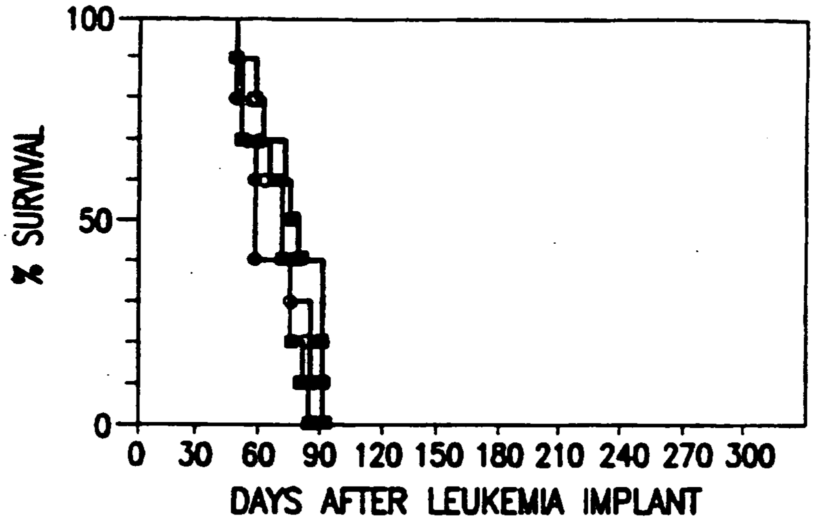


FIG.3B


100
50
0
% SURVIVAL
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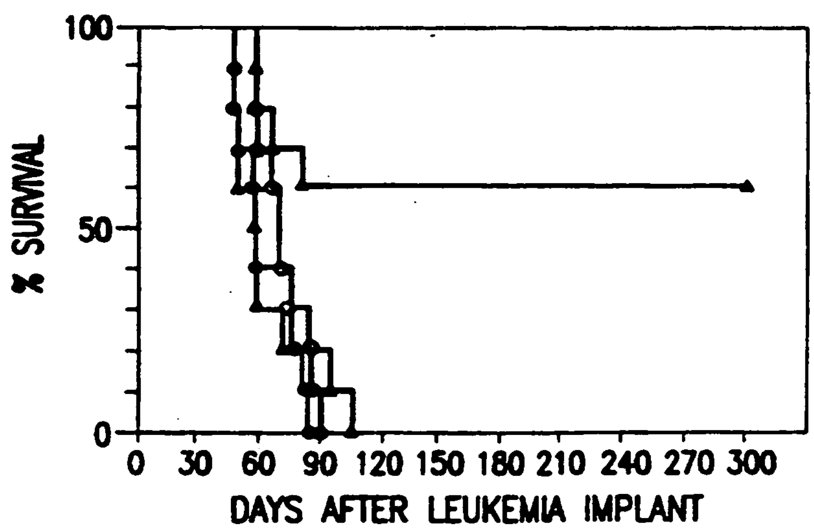
DAYS AFTER LEUKEMIA IMPLANT

FIG.4A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50
0
% SURVIVAL
0 30 60 90 120 150 180 210 240 270 300
DAYS AFTER LEUKEMIA IMPLANT

FIG.4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17320

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C07H 21/04; C12Q 1/68
US CL :514/44; 536/24.5; 435/6, 91.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/24.5, 435/6, 91.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog: Medline, BIOSIS, Derwent Biotechnology Abstracts, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	NAKAI et al. Cellular uptake Mechanism for oligonucleotides: involvement of endocytosis in the uptake of phosphodiester oligonucleotides by a human colorectal adenocarcinoma cell line, HCT-15. Journal of Pharmacology and Experimental Therapeutics 1996, Vol. 276, No. 3, pages 1362-1372, see entire document.	1-21 ------ 1-21
X --- Y	LIU et al. Modulation of multidrug resistance gene (*mdr-1*) with antisense oligonucleotides. Clinical Science. 1996, Vol. 91, pages 93-98, see entire document.	1-21 ------ 1-21
X --- Y	BERTRUM et al. Reversal of multiple drug resistance *in vitro* by phosphorothioate oligonucleotides and ribozymes. Anti-Cancer Drugs. 1995, Vol. 6, pages 124-134, see entire document.	1-21 ------ 1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

02 DECEMBER 1997

Date of mailing of the international search report

23 DEC 1997

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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	THIERRY et al Overcoming multiple drug resistance in human tumor cells using free and liposomally encapsulated antisense oligonucleotides. Biochemical and Biophysical Research Communications. 15 February 1993, Vol. 190, No. 3, pages 952-960, see entire document.	1-21 ------ 1-21
X --- Y	RAMACHANDRAN et al. Reversal of multidrug resistance by MDR-1 antisense phosphorothioate oligodeoxy nucleotides in SW620 Ad300 human colon carcinoma cells in vitro in xenografts. Proceedings of the American Association for Cancer Research. March 1995, Volume 36, page 412, Abstract 2456, see entire abstract.	1-21 ------ 1-21
X --- Y	WO 96/02556 A2 (HYBRIDON, INC.) 01 February 1996, see entire document.	1-21 ------ 1-21